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(54) Title: BACTERICIDAL AND/OR BACTERIOSTATIC PEPTIDE ISOLATED FROM COLEOPTERAN INSECTS

(57) Abstract

An antibacterial factor inducible in the haemolymph of a Coleopteran insect by a bacterial infection.

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BACTERICIDAL AND/OR BACTERIOSTATIC PEPTIDES ISOLATED FROM COLEOPTERAN INSECTS

Background of the Invention

This invention relates to new bactericidal and/or bacteriostatic peptides isolated from the haemolymph of Coleopteran insects. This invention also relates to a process for the isolation of the peptides and to processes for their use.

Insects are among the oldest groups in the animal kingdom. Their omnipresence nowadays and their way of life in numerous groups found in small spaces suggest that they have developed effective defense systems against infections.

It is known that at least several orders of insects possess antibacterial factors which appear in their haemolymph in response to bacterial infection of the haemocoel (FRIES-1984; ref. 1). The cellular response, mediated by the insects haemocytes, enables them to eliminate almost immediately the foreign material from the haemocoel by phagocytosis, nodule formation and encapsulation. Their cellular defense system is assisted by the humoral (cell-free) response which produces humoral antibacterial factors which kill remaining bacteria and protect the insect against subsequent bacterial challenge (DUNN-1986; ref. 2). Such humoral factors have received much attention during recent years.

Besides some factors with unknown function, such as P4 from Hyalophora cecropia (ANDERSON-1987; ref. 3), three families of bactericidal proteins have been isolated from both larval and pupal stages of different insect species : lysozymes, cecropins and attacins.

The lysozymes are basic proteins with properties similar to those of chicken egg white lysozyme

(DUNN-1986; ref. 2) and with molecular weights of about 15 300 - 16 200.

Cecropins are basic peptides with molecular weights of about 3500 - 4 000. They have been isolated from several Lepidopteran insects and reviewed in BOMAN et al, 1986 (ref. 4). Such a peptide, Sarcotoxin I, is also the subject of European patent publication 0182278 (Wakunaga Seiyaku KK)

Attacins are proteins with molecular weights of about 20 000 - 23 000. Six related components were isolated from H. cecropia (BOMAN et al 1986; ref. 4). Attacin-like proteins were also found in Manduca sexta (HURLBERT et al-1985; ref. 5).

Recently, cecropin- and attacin-like substances have also been reported in Glossina morsitans (KAAYA et al-1986; ref. 6) and in Celerio euphorbia (JAROSZ-1986; ref. 7). Other bactericidal peptides from insects have been reported, such as: the diptericins which are inducible immune proteins from the dipteran, Phormia terranovae (KEPPI et al - 1986 ref. 14; DIMARCQ et al - 1988; ref. 15); the bactericidal inducible immune compounds from the fruitfly, Ceratitis capitata (Postlethwait et al - 1988; ref. 16); and defensin (sapecin) from flies (MATSUYAMA et al - 1988; ref 13; LAMBERT et al - 1989; ref 10). Broad host range antibiotic peptides (the so-called "Magainins") have also been isolated from the frog, Xenopus laevis (ZASLOFF et al - 1988; ref. 17). More recently, bacterial peptides from the immune haemolymph of Hymenoptera, especially honeybees such as apidaecins, abaecins, hymenoptaecins, and hymenaecins, consisting of, respectively, 10 to 20 residues, 30 to 40 residues, 80 to 100 residues, and 55 to 60 residues, have been described in European patent application 88 401673.4.

These known proteins often seem to have a broad-spectrum bactericidal action against one or more bacteria. For example, lysozymes have bactericidal effect against certain bacteria of Gram+ type.

Summary of the Invention

In accordance with this invention is provided novel antibacterial factors which can be induced in the haemolymph of Coleopteran insects, preferably Tenebrio molitor and Leptinotarsa decemlineata. Most of these antibacterial factors comprise peptides with novel bactericidal and/or bacteriostatic properties not currently possessed by already known antibacterial factors, inducible in other insects.

Detailed Description of the Invention

The bacteriostatic and/or bactericidal factors of this invention are isolatable from the haemolymph ("lymph") of immune Coleopteran insects, preferably Tenebrio molitor and Leptinotarsa decemlineata. By "immune" is meant that the Coleopteran insects have been injected with a foreign material, such as viable bacteria (e.g., E. coli) or particles having the same antigenic properties, so as to induce the production therein of a number of factors, particularly peptides, which: 1) have bactericidal or bacteriostatic properties; 2) can be isolated from the haemolymph of such injected Coleopteran insects; and 3) are not present in Coleopteran insects which have not received such an injection.

The antibacterial factors of this invention, obtained from immune Coleoptera, comprise one or more peptides which are not present in the haemolymph of non-immune Coleopteran insects and which are different from lysozymes, cecropins, diptericins, magainins,

attacins, apidaecins, abaecins, hymenoptaecins and hymenaecins with respect to their activity spectrum and their amino acid composition. Preferred antibacterial factors of this invention are virtually free of peptides present in the haemolymph of non-immune Coleopteran insects and of lysozymes, cecropins, diptericins, magainins, attacins, apidaecins, abaecins, hymenoptaecins and hymenaecins. However, like such peptides, the factors of this invention are also "thermostable" in that their antibacterial properties are not affected by a thermal treatment at about 100°C over a period of about 5 minutes.

The factors of this invention are present in haemolymph compositions of immune Coleopteran insects, preferably Tenebrio molitor and Leptinotarsa decemlineata. Such compositions, as obtained from immune Coleoptera, can be purified in a conventional manner, for example by a thermal treatment, particularly at about 100°C for about 5 minutes, in order to obtain the compositions virtually free of thermolabile proteins.

The different factors present in the purified compositions can then be separated from each other and isolated using reversed phase high performance liquid chromatography ("HPLC") (CASTEELS et al-1990; ref. 18). In this regard, the individual factors can be isolated in a conventional manner, such as by absorption from a dilute solution (e.g., 0.1%) of a polar solvent (e.g., trifluoroacetic acid) on a non-polar stationary phase formed by spherical beads of silica carrying, for example, either C4-aliphatic, C18-aliphatic or di-phenyl ligands covalently attached to the silica. An example of such a non-polar stationary phase is a column packing for reversed phase HPLC commercialized under the designation VYDAC 214 TP 54, VYDAC 218 TP 54

or VYDAC 219 TP 54, respectively (CASTEELS et al - 1990; ref. 18). The individual factors can subsequently be desorbed from the non-polar stationary phase by elution with a gradient of progressively increased concentration of a solution of acetonitrile (70%) in a solvent such as the dilute solution of the polar solvent, particularly when the acetonitrile concentration, initially at a lower concentration, reaches a value ranging from about 5% to about 40% by volume.

Preferably, the HPLC is carried out with a non-polar stationary phase (column packing) which absorbs polypeptides by a hydrophobic interaction. This interaction can be disrupted by increasing the percentage of organic solvent ("solution B") in the aqueous mobile phase ("solution A"). Preferably, solution A consists of 0.1% trifluoroacetic acid in water and solution B consists of 70% acetonitrile in solution A. Acetonitrile is preferably added regularly and progressively at a flow rate of 1 ml/minute. Preferably, the aqueous solution A has a low pH (e.g., pH 2) so that the alpha-amino groups and basic amino acid side chains are protonated, rendering the peptides less polar and resulting in an increased retention of especially basic and neutral peptides.

Conventional silica-based column packings can be used, such as those which consist of hydrocarbon ligands covalently attached to the surface of spherical silica beads. However, other packings can be used for isolating the antibacterial factors of this invention. Preferred silica-based packings include: C4 column packings, in which the ligands consist of C4 hydrocarbon chains and which offer a good resolution for small proteins of up to 100 kilodaltons; C18 column packings having a high selectivity for small peptides

of up to 20 residues; and packings comprising diphenyl ligands which are about equal in hydrophobicity to C4 chains but offer a better absorption of aromatic residues.

The peptides of the invention have been found to possess bactericidal properties or bacteriostatic properties, or both, against various bacteria, especially against Gram- bacteria, such as Erwinia, Salmonella and Pseudomonas. The peptides can be formulated in a conventional manner into compositions for use in fields such as antibacterial therapy and prophilaxy in man or animal, plant pathogen control and preservation of food, feed and cosmetics. Such compositions can contain one or more peptides of this invention, in combination with a suitable (e.g., pharmaceutically acceptable) carrier, at a dose sufficient for the expression of bactericidal and/or bacteriostatic properties.

It goes without saying that the antibacterial peptides of this invention (such as the preferred peptides "coleo 1" to "coleo 15" isolated in the following Examples) are not limited to specific peptides isolated from the haemolymph of immune Coleopteran insects. Such peptides can also be synthetically prepared, particularly by conventional chemical processes or recombinant DNA techniques. Also, any equivalent antibacterial peptides, that differ from the preferred peptides described in the following Examples, come within the invention, such as peptides modified by the substitution of some amino acids or by shortening or elongating the peptides, provided such modifications do not alter the essential bactericidal and/or bacteriostatic properties of the peptides.

Genes encoding the antibacterial peptides of this invention can be stably inserted in a conventional

manner into the nuclear genome of a plant, using the procedures described, for example, in PCT publication WO 88/00976 (which is incorporated herein by reference). In this regard, a gene encoding the peptide can be suitably inserted: downstream of, and under the control of, a promoter which can direct the expression of the gene in plant cells; and upstream of suitable 3' transcription regulation signals (i.e., transcript 3'-end formation and polyadenylation signals).

Other characteristics and advantages of this invention will become apparent in the light of the following Examples. The figures, referred to in the Examples, are as follows:

- figure 1 shows the reverse phase analysis of an immune haemolymph compared to a non-immune haemolymph ("control"), both isolated from Tenebrio molitor larvae on a C_4 column (gradient 0-50% B in 50 minutes, 50-70% B in 10 min and 70-100% B in 8 min; flow : 1 ml/min) where:

1 = coleo 1
2 = coleo 2a and coleo 2b
3 = coleo 3
4 = coleo 4
5 = coleo 5.

- figure 2 shows the reverse phase analysis of an immune haemolymph compared to a non-immune haemolymph ("control"), both isolated from Tenebrio molitor adults on a C4 column (gradient 0-50% B in 50 minutes, 50-70% B in 10 min and 70-100% B in 8 min; flow 1 ml/min) where:

6 = coleo 6
7 = coleo 7
8 = coleo 8.

- figure 3 shows the reverse phase analysis of an immune haemolymph compared to a non-immune haemolymph

("control"), both isolated from Leptinotarsa decemlineata larvae on a C4 column (gradient 0-50% B in 50 minutes, 50-70% B in 10 min and 70-100% B in 8 min; flow 1 ml/min) where:

9 = coleo 9
10 = coleo 10
11 = coleo 11
12 = coleo 12.

- figure 4 shows the reverse phase analysis of an immune haemolymph compared to a non-immune haemolymph ("control"), both isolated from Leptinotarsa decemlineata adults on a C4 column (gradient 0-50% B in 50 minutes, 50-70% B in 10 min and 70-100% B in 8 min; flow 1 ml/min) where:

13 = coleo 13
14 = coleo 14
15 = coleo 15.

- figure 5 represents the chromatogram showing the further resolution of the coleo 2 peak, obtained from the C4 chromatography, into two distinct peaks (indicated as 2a and 2b) on a C18 column (gradient 25-100% B in 50 minutes; flow 1 ml/min) where:

2a = coleo 2a
2b = coleo 2b.

- figure 6 represents the chromatogram showing the further resolution of the coleo 10 peak, obtained from the C4 chromatography, into two distinct peaks (indicated as 10a and 10b) on a C18 column (gradient 20-60% B in 40 minutes; flow 1 ml/min).

EXAMPLES

I MATERIALS AND METHODS

Insects:

Tenebrio molitor and Leptinotarsa decemlineata were raised under standard conditions.

Bacteria :

The following assay organisms : E. coli NCTC 9001, Xanthomonas campestris pv. vesicatoria LMG905, Pseudomonas syringae pv. tabaci NCPPB1302, Pseudomonas syringae pv. tomato NCPPB1106, Erwinia carotovora pv. atroseptica LMG2378, Erwinia salicis NCPPB2530, Erwinia carotovora pv. carotovora NCPPB312; Serratia marcescens ATCC17991, Corynebacterium michiganense pv. michiganense NCPPB1573 and Pseudomonas aeruginosa CCEB481, were used for screening the antibacterial activity of different humoral factors from immune haemolymph ("lymph"). These strains were grown aerobically at 28°C on agarplates (NA or 10% TSA).

NA is a nutrient agar commercially available from the Company: DIFCO of Detroit, M.I. (USA). TSA (Trypticase Soy Agar) is commercially available from the Company: BBC of Cockseysville (USA).

Bacterial suspensions were made in PBS (phosphate buffered saline : 0.8% NaCl, 0.02% KH_2PO_4, 0.02% KCL, 0.115% Na_2HPO_4) 0.15M, pH = 7.2. Bacterial concentrations were determined in a counting chamber; living suspensions were verified by plate counts.

II PREPARATION OF IMMUNE HEMOLYMPH OF COLEOPTERAN INSECTS.

Immunisation procedure

The Coleopteran insects, Tenebrio molitor and Leptinotarsa decemlineata, were anesthetized with CO_2 and were infected with a sublethal dose of E. coli. In this regard, they were intrahaemocoelic injected under sterile conditions with 10^4 to 10^5 E. coli NCTC 9001 suspended in 1 ul PBS.

When large lymph samples were required, the insects were infected by wounding them with a needle previously submerged in the E.coli suspension.

Haemolymph preparation

Two days after the immunisation, the immune and control haemolymphs were obtained from anesthetised insects by puncturing the intersegmental membrane of the abdomen with a heat-sharpened glass capillary.

The haemolymph was pooled in an ice-cooled tube containing a few crystals of phenylthiourea ("PTU") to prevent melanisation of the haemolymph, was diluted with an equal volume of PBS (ph 7.2), and was used immediately or stored frozen at -70°C. The preparation obtained provided the "immune lymph", referred to hereinafter.

III ANTIBACTERIAL ASSAY OF TOTAL IMMUNE LYMPH : AGAR DIFFUSION ASSAY

For the test, 9cm Petri dishes were inoculated with a bacterial suspension (10^4 cells/ml) in H_2O. Growth medium was Nutrient agar (E.c., S.m., C.m.) or 10% TSA (E.c.a., E.c.c., E.s., P.a., P.st, Pst, Xc). H_2O was evaporated under a laminar flow. Wells with a radius of 2mm were made for application of samples. The samples were boiled for 5 minutes before use and centrifuged at 13 000 rpm, 5' in Eppendorf centrifuge. 25 ul of the supernatant was added to the wells.

Growth inhibition was assessed when bacterial growth was clearly visible (between 12-48 hours after inoculation). Determination of the growth inhibition was done by measurement of the radius of the growth inhibition zone (mm). The radius of the wells (2mm) was subtracted.

Properties of the humoral factors induced in the haemolymph of immune Coleopteran insects

It was found, as discussed hereinafter, that the sublethal dose of E. coli bacteria had induced in the immunized Coleopteran insects several factors (not present in control Coleopteran insects) which protect the insects against subsequent bacterial infection. Immune lymph was shown to be bactericidal against E.coli.

The humoral factor, directed against E.coli NCTC 9001, was heat resistant since boiling for 5 minutes did not affect its bactericidal activity.

Immune lymph from E.coli-vaccinated Tenebrio molitor and Leptinotarsa decemlineata was also bactericidal against some other strains, indicating that the humoral response was not specific.

The humoral response was not only activated by injecting viable bacteria. Heat-killed bacteria and inert particles, such as chinese ink and latex beads, were also found to elicit a similar activity.

IV PURIFICATION OF HUMORAL FACTORS BY REVERSED PHASE HPLC

The immune lymph was first purified by boiling at 100°C during 5 minutes.

The heated lymphs from non-immune or blank (B) and immune (I) Coleopteran insects then were further purified by a reversed phase high performance liquid chromatography system (Kratos of Ramsey, NJ, USA). As a precolumn was used the commercially available Uptight precolumn part no. 35-B (Upchurch Scientific, Inc.), and thereafter were used, for further separations and purifications, standard (250 x 4.6 mm) C4, C18 and diphenyl columns, commercially available under the designations VYDAC 214 TP 54, VYDAC 218 TP 54 and VYDAC 219 TP 54, respectively (The Separation Group of Hesperia, CA, U.S.A). Solvent A (0.1% trifluoroacetic

acid ("TFA") in water) was initially added to the lymph samples to a final concentration of 50% (V/V), and the resulting solutions were applied to a Kratos HPLC system containing The Separation Group C4 column and eluted in solvent B (70% acetonitrile ("MeCN") in solvent A) with a gradient of 0.7% MeCN/min at a flow rate of 1 ml/min.

Figures 1 to 4 show UV absorbance of the blank lymph (control) versus the immune lymph and the slanted dotted line in each figure represents the variation of the concentration of solution B (70% MeCN) in the gradients formed. UV detection was performed at a wavelength of 214 nm for smaller peptides and 280 nm for larger proteins.

Chromatography of the immune lymph of Tenebrio larvae revealed 5 active peaks (figure 1); immune lymph of Tenebrio adults revealed 3 active peaks (figure 2); immune lymph of Leptinotarsa larvae revealed 4 active peaks (figure 3); and immune lymph of Leptinotarsa adults revealed 3 peaks (figure 4). None of these peaks was present in the blank lymph (control). For each insect tested, the antibacterial factors (labelled by numbers above the corresponding peaks in figures 1-4), were essentially formed of peptides, hereinafter respectively designated as "coleo 1" to "coleo 15", which were all collected.

A first factor ("coleo 1"), isolated from immune Tenebrio molitor larvae, was desorbed at a concentration of about 40.2% solution B.

A second factor ("coleo 2"), isolated from immune Tenebrio molitor larvae, was desorbed at a concentration of about 40.6% solution B.

A third factor ("coleo 3"), isolated from immune Tenebrio molitor larvae, was desorbed at a concentration of about 43.3% solution B.

A fourth factor ("coleo 4"), isolated from immune Tenebrio molitor larvae, was desorbed at a concentration of about 43.8% solution B.

A fifth factor ("coleo 5"), isolated from immune Tenebrio molitor larvae, was desorbed at a concentration of about 45% solution B.

A sixth factor ("coleo 6"), isolated from immune Tenebrio molitor adults, was desorbed at a concentration of about 36.8% solution B.

A seventh factor ("coleo 7"), isolated from immune Tenebrio molitor adults, was desorbed at a concentration of about 40.2% solution B.

A eight factor ("coleo 8"), isolated from immune Tenebrio molitor adults, was desorbed at a concentration of about 40.6% solution B.

A ninth factor ("coleo 9"), isolated from immune Leptinotarsa decemlineata larvae, was desorbed at a concentration of about 15.8% solution B.

A tenth factor ("coleo 10"), isolated from immune Leptinotarsa decemlineata larvae, was desorbed at a concentration of about 41.2% solution B.

An eleventh factor ("coleo 11"), isolated from immune Leptinotarsa decemlineata larvae, was desorbed at a concentration of about 42.2% solution B.

A twelfth factor ("coleo 12"), isolated from immune Leptinotarsa decemlineata larvae, was desorbed at a concentration of about 42.6% solution B.

A thirteenth factor ("coleo 13"), isolated from immune Leptinotarsa decemlineata adults, was desorbed at a concentration of about 15.8% solution B.

A fourteenth factor ("coleo 14"), isolated from immune Leptinotarsa decemlineata adults, was desorbed at a concentration of about 43.3% solution B.

A fifteenth factor ("coleo 15"), isolated from immune Leptinotarsa decemlineata adults, was desorbed at a concentration of about 44% solution B.

Using HPLC under the same conditions on a C-18 column, coleo 2 could, after redilution in solution A, be separated into two components (figure 5): "coleo 2a", the desorption of which occurs at a concentration of about 48.8% solution B, and "coleo 2b", the desorption of which occurs at a concentration of about 49.5% solution B.

Using HPLC under the same conditions on a C-18 column, the desorption of coleo 1 occurs at a concentration of about 48% solution B.

Using HPLC under the same conditions on a C-18 column, the desorption of coleo 3 occurs at a concentration of about 46.8% solution B.

Using HPLC under the same conditions on a C-18 column, the desorption of coleo 4 occurs at a concentration of about 52.4% solution B.

Using HPLC under the same conditions on a C-18 column, the desorption of coleo 5 occurs at a concentration of about 52.8% solution B.

Using HPLC under the same conditions on a C-18 column, coleo 10 could, after redilution in solution A, be separated into two components (figure 6): "coleo 10a", the desorption of which occurs at a concentration of about 43.2% solution B and "coleo 10b", the desorption of which occurs at a concentration of about 43.6% solution B.

Using HPLC under the same conditions on a C-18 column, the de-sorption of coleo 11 occurs at a concentration of about 41.4% solution B.

Using HPLC under the same conditions on a C-18 column, the de-sorption of coleo 12 occurs at a concentration of about 45.4% solution B.

Using HPLC under the same conditions on a C-18 column, the de-sorption of coleo 14 occurs at a concentration of about 45.5% solution B.

Using HPLC under the same conditions on a C-18 column, the de-sorption of coleo 15 occurs at a concentration of about 46% solution B.

Using HPLC under the same conditions on a diphenyl column, the desorption of coleo 9 occurs at a concentration of about 17% solution B.

Among these antibacterial factors (coleo 1-15) of this invention, inducible in the haemolymph of Coleoptera, preferred factors comprise coleo 1, 2a, 2b, 3, 4, 6, 9 and 15.

The relative proportion of a number of amino acids in the amino acid compositions of some preferred antibacterial peptides of this invention has been determined, using both post-column O-phtaldialdehyde derivatization (BENSON and HARE - 1975; ref. 9) and pre-column phenylisothiocyanate derivatization (BIDLINGMEYER et al - 1984; ref. 11) after 24h and 72h total hydrolysis (4 analyses per sample). The results, which follow, are to be understood as % (measured) of each of the designated amino acids with respect to all of the amino acids which gave rise to similar measurements. Hence, some amino acids may not have been taken into account in the "100% total", with respect to which the percentage values have been indicated. That could be the case, for example, in relation to cysteine and tryptophane, if present. However some amino acids have some times been deemed to be absent. Those amino acids are then followed by a "slash" sign ("/").

In the following;

- Asx means either Asn or Asp, and
- Glx means either Gln or Glu.

A particularly preferred coleo 1 ("coleo 1'") comprises the following relative proportions of the following amino acid residues:

Asx: 13.8
Glx: 7.9
Ser: 9.1
Gly: 15.2
His: 4.7
Arg: 5.2
Thr: 5.5
Ala: 4.5
Pro: 6.0
Tyr: 3.5
Val: 6.4
Met: /
Ile: 2.4
Leu: 4.7
Phe: 4.4
Lys: 6.6

A particularly preferred coleo 2a ("coleo 2a'") comprises the following relative proportions of the following amino acid residues:

Asx: 11.4
Glx: 9.4
Ser: 8.7
Gly: 10.6
His: 3.1
Arg: 7.4
Thr: 4.9
Ala: 5.2
Pro: 5.1
Tyr: 3.7
Val: 4.8
Met: /
Ile: 7.2

Leu: 7.9
Phe: 4.0
Lys: 6.7

A particularly preferred coleo 2b ("coleo 2b'") comprises the following relative proportions of the following amino acid residues:

Asx: 11.3
Glx: 10.6
Ser: 9.1
Gly: 16.5
His: 3.6
Arg: 6.2
Thr: 5.1
Ala: 5.1
Pro: 7.1
Tyr: 2.3
Val: 6.9
Met: /
Ile: 2.3
Leu: 4.4
Phe: 4.5
Lys: 5.0

A particularly preferred coleo 3 ("coleo 3'") comprises the following relative proportions of the following amino acid residues:

Asx: 12.9
Glx: 15.2
Ser: 8.7
Gly: 4.5
His: 4.5
Arg: 4.5
Thr: 4.2
Ala: 7.2
Pro: 5.3
Tyr: /

Val: 6.8
Met: /
Ile: 3.0
Leu: 5.7
Phe: 5.3
Lys: 12.1

A particularly preferred coleo 4 ("coleo 4'") comprises the following relative proportions of the following amino acid residues:

Asx: 10
Glx: 14.3
Ser: 8.0
Gly: 10.0
His: 4.0
Arg: 4.7
Thr: 3.7
Ala: 8.7
Pro: 7.0
Tyr: /
Val: 6.4
Met: /
Ile: 3.0
Leu: 5.7
Phe: 4.7
Lys: 9.7

A particularly preferred coleo 6 ("coleo 6'") comprises the following relative proportions of the following amino acid residues:

Asx: 17.1
Glx: 6.8
Ser: 6.5
Gly: 19.5
His: 4.2
Arg: 9.6
Thr: 2.3

Ala: 4.4
Pro: 7.5
Tyr: 1.0
Val: 7.5
Met: /
Ile: 3.7
Leu: 2.9
Phe: 2.3
Lys: 5.2

A particularly preferred coleo 9 ("coleo 9'") comprises the following relative proportions of the following amino acid residues:

Asx: 10.7
Glx: 3.6
Ser: 17.8
Gly: 36.0
His: 3.0
Arg: 6.6
Thr: 4.1
Ala: 5.6
Pro: 4.1
Tyr: 2.0
Val: 1.5
Met: /
Ile: /
Leu: 2.0
Phe: /
Lys: 2.0

and has the following amino acid sequences at its N-terminal end :

GKNAGPHGANRGSSSGGGSNRGGSNR

which has been determined using an ABI 470A (Applied Biosystems Inc., Foster City, CA, U.S.A.) automized gas-phase sequencer (HEWICK et al - 1981; ref 8) and an ABI 120A on line phenylthiohydantoin ("PTH") amino acid

analyzer. Stepwise liberated PTH-amino acids were quantified with a D2000 chromato-integrator (Hitachi).

A particularly preferred coleo 15 ("coleo 15'") comprises the following relative proportions of the following amino acid residues:

Asx: 17.4
Glx: 8.5
Ser: 4.7
Gly: 13.8
His: 5.0
Arg: 4.6
Thr: 4.9
Ala: 6.0
Pro: 7.8
Tyr: 1.9
Val: 7.5
Met: /
Ile: 4.3
Leu: 3.6
Phe: 3.4
Lys: 6.7

The humoral response was activated in a similar fashion when Leptinotarsa and Tenebrio were injected with chinese ink and latex beads. Immune lymph from such insects also exhibited the above-mentioned peaks (though to a smaller extent) which were not present in the lymph of blank insects. This also confirmed that such peaks were not products of bacterial lysis.

Antibacterial assay of total immune lymph

Bactericidal activity of the total immune lymph of Leptinotarsa and Tenebrio larvae and adults has been shown by its capabability of inhibiting bacterial growth in the agar diffusion assay (POSTLETHWAIT et al-1988; ref. 16). The results, summarized in Table I, below, each show the radius (mm) of inhibition of

bacterial growth by each antibacterial lymph minus the radius of the application well containing the lymph.

Table I

	L/a	L/l	T/a	T/l
Escherichia coli NCTC 9001	3	3.5	4	4
Serratia marcescens ATCC 17991	3	3	1.5	1
Corynebacterium michiganense pv. michiganense NCPPB 1573	3	3.5	0	0
Erwinia carotovora pv. atroseptica LMG 2378	6	4	6	6
Erwinia carotovora pv. carotovora NCPPB 312	4	3	6	6
Erwinia salicis NCPPB 2530	7	3	4.5	4.5
Pseudomonas aeruginosa CCEB 481	0.5	0	0.5	0.5
Pseudomonas syringae pv. tabaci NCPPB 1302	8	3	5.5	5
Pseudomonas syringae pv. tomato NCPPB 1106	8	2	9	5.5
Xanthomonas campestris pv. vesicatoria LMG 905	1	0	2* 6 9.5	3

L Leptinotarsa decemlineata; T = Tenebrio molitor;
a = adult; l = larval
* Different concentric rings with different degrees of growth inhibition were visible.

Antibacterial assay of purified immune peptides

The bactericidal activity of all the peptides (coleo 1-coleo 15), purified on the C4 column above, has been shown by their capability of inhibiting the growth of several bacterial strains (European patent publication 0299828) as indicated in Table II, below.

Immune lymph was collected from immunized Coleoptera and diluted immediately with PBS (1/1 by volume). Small amounts of PTU were added to prevent oxidation. The samples were stored at -70°C. The samples were then thawed, boiled for 5 min. and centrifuged for 5 min. at 13 000 rpm in an Eppendorf centrifuge. A given volume of this material was subjected to HPLC separation. Peaks were collected, and the eluent was evaporated under vacuum (Speedvac, Savant Instruments of Farmingdale, NY, U.S.A). The material from each peak was then dissolved in the same volume of H_2O as the original sample volume (immune lymph+PBS).

The assay was performed in 96 well plates. The wells were filled with 50 ul of liquid medium (Nutrient broth 10% for E.coli, S.marcescens and C.michiganense and TSB 10% for the other bacteria), 50 ul of a suspension of bacterial cells in H_2O (10^4cells/ml) and 25 ul of the sample or of a dilution thereof. Each sample was tested undiluted and 2.5, 5, 10 and 25 times diluted. Growth of the bacteria was visually assessed after 24h.

Table II

Bacterium	1	2a	2b	3	4	5	6	7	8	9	10a	10b	11	12	13	14	15
E. coli	5	-	-	-	-	-	2.5	5	5	1	-	2.5	-	-	1	-	1
S. marcescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. michiganense	N	N	N	N	N	N	N	N	N	-	N	N	N	N	-	-	-
E. car. atr.	10	-	-	-	-	-	2.5	5	5	-	2.5	-	1	2.5	25	25	25
E. car. car.	-	-	-	-	-	-	-	-	-	25	-	-	-	-	25	-	-
E. salicis	25	10	25	25	25	25	5	25	25	25	2.5	10	2.5	2.5	25	25	25
P. aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. syr. pv. tab.	5	-	-	2.5	-	-	1	2.5	5	-	-	1	-	-	-	-	-
P. syr. pv. tom.	10	-	-	10	-	25	1	2.5	5	-	-	-	-	-	-	-	-
X. campestris	-	-	-	2.5	-	2.5	-	-	-	-	-	-	-	-	-	-	-

In Table II, the highest sample dilution at which bacterial growth was prevented is indicated (a higher value indicates that bacterial growth was inhibited with a more diluted sample.) "N" means not tested, and "-" means no activity.

The preceeding results show that this invention provides numerous bactericidal peptides active against Gram-bacteria. Depending on the microorganism to be destroyed or inhibited, one may use either the most effective peptide or mixtures of the relevant peptides, particularly when the control of several microorganisms is sought at the same time. A composition containing a bactericidally and/or bacteriostaticaly effective amount of one or several of such peptides can be suitably utilized in a conventional manner against pathogenic bacteria, for example in human and animal therapy and prophilaxy, in plant pathogen control, and in preserving footstuffs and cosmetics. In this regard, such compositions can be formulated in a conventional manner to be applied topically in the form of a solution or spray or orally or parenterally. Such a composition can also be formulated in a conventional manner to be: applied to plants, incorporated in human food or animal feed or cosmetics, applied as a liquid or solid distributable over large acreages or added in chemical, food and feed production processes.

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Claims

1. An antibacterial factor inducible in the haemolymph of a Coleopteran insect, such as by a bacterial infection.

2. The factor of claim 1 which is selected from the group consisting of coleo 1 to coleo 15.

3. The factor of claim 1 or 2 which is selected from the group consisting of coleo 2a, coleo 2b, coleo 10a and coleo 10b.

4. The factor of claim 1 or 2 which is selected from the groups consisting of coleo 1, coleo 2a, coleo 2b, coleo 3, coleo 4, coleo 6, coleo 9 and coleo 15, particularly coleo 1', coleo 2a', coleo 2b', coleo 3', coleo 4', coleo 6', coleo 9' and coleo 15'.

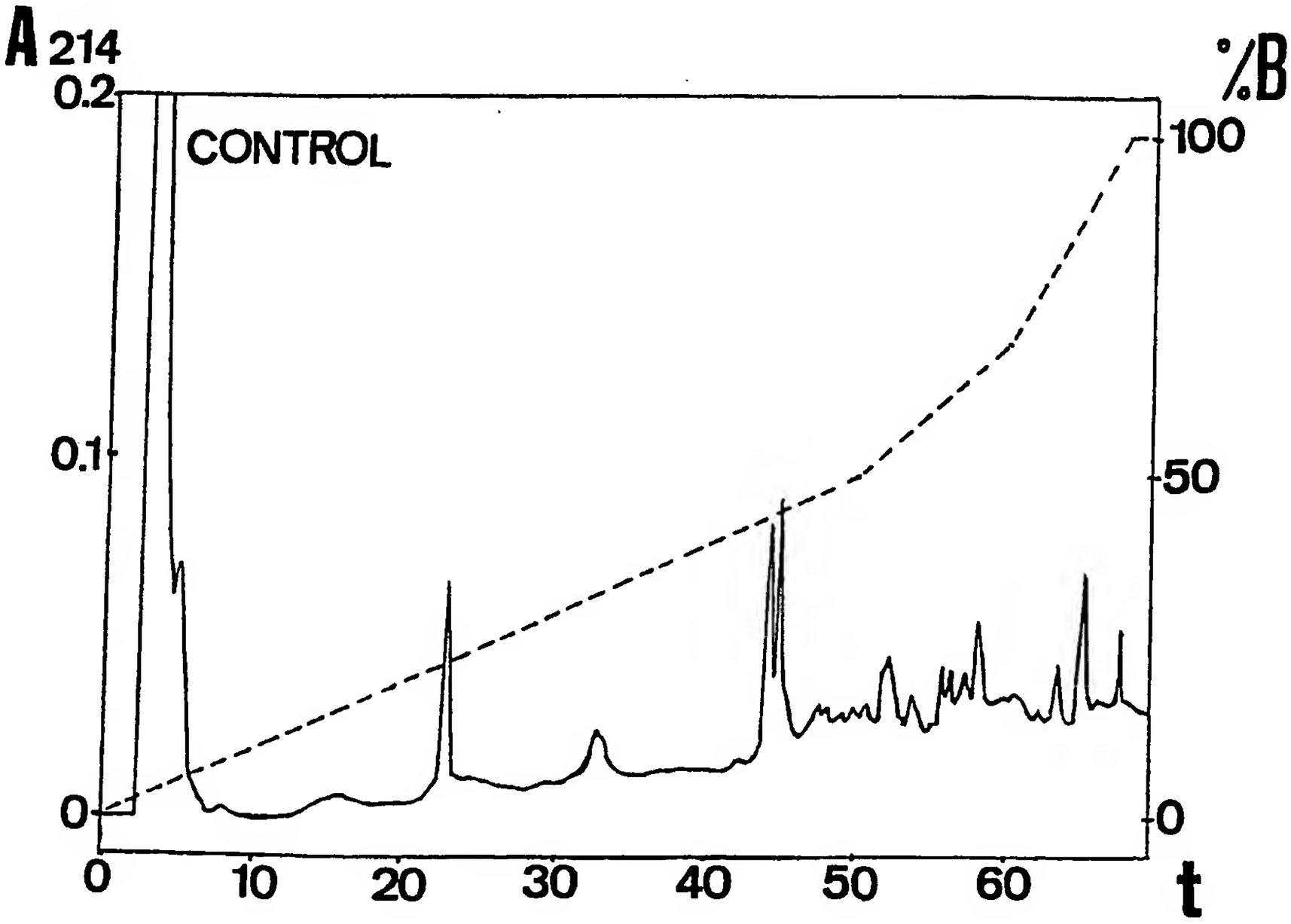
5. A bacteriocidal and/or bacteriostatic composition comprising an effective amount of the factor of anyone of claims 1-4 and a vehicle.

6. A method for combatting a bacterial infection of a host, comprising the step of applying to the host a bacteriocidally or bacteriostatically effective amount of the factor of anyone of claims 1-4.

7. A method for preserving a foodstuff or a cosmetic comprising the step of incorporating therein an antibacterial-effective amount of the factor of anyone of claims 1-4.

8. A method of rendering a plant resistant to a bacterial plant pathogen, comprising the step of: transforming the plant with a DNA sequence encoding the factor of anyone of claims 1-4; the DNA sequence being in the same transcriptional unit as, and under the control of, a promoter capable of directing expression of the DNA sequence in cells of the plant.

FIG 1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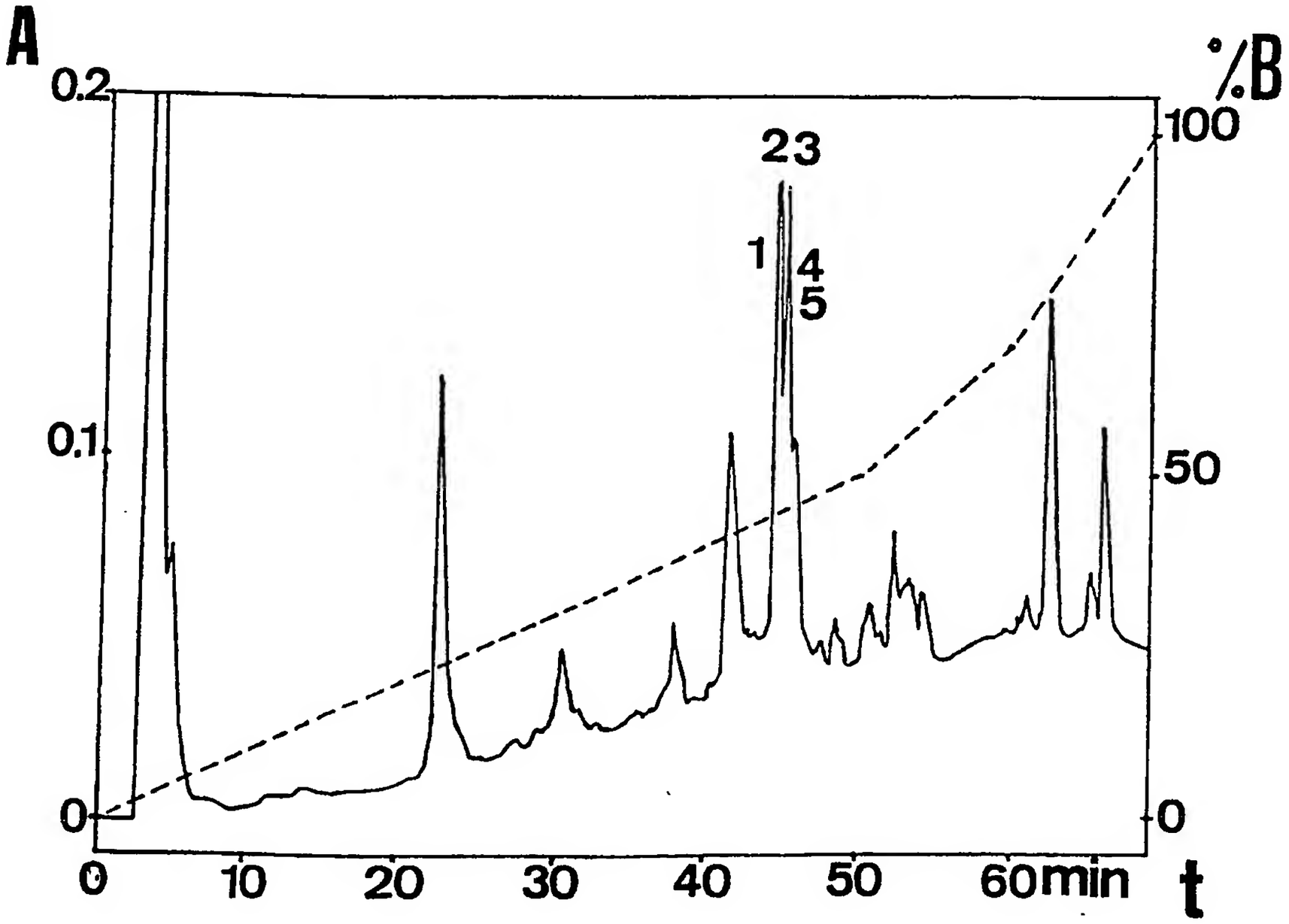
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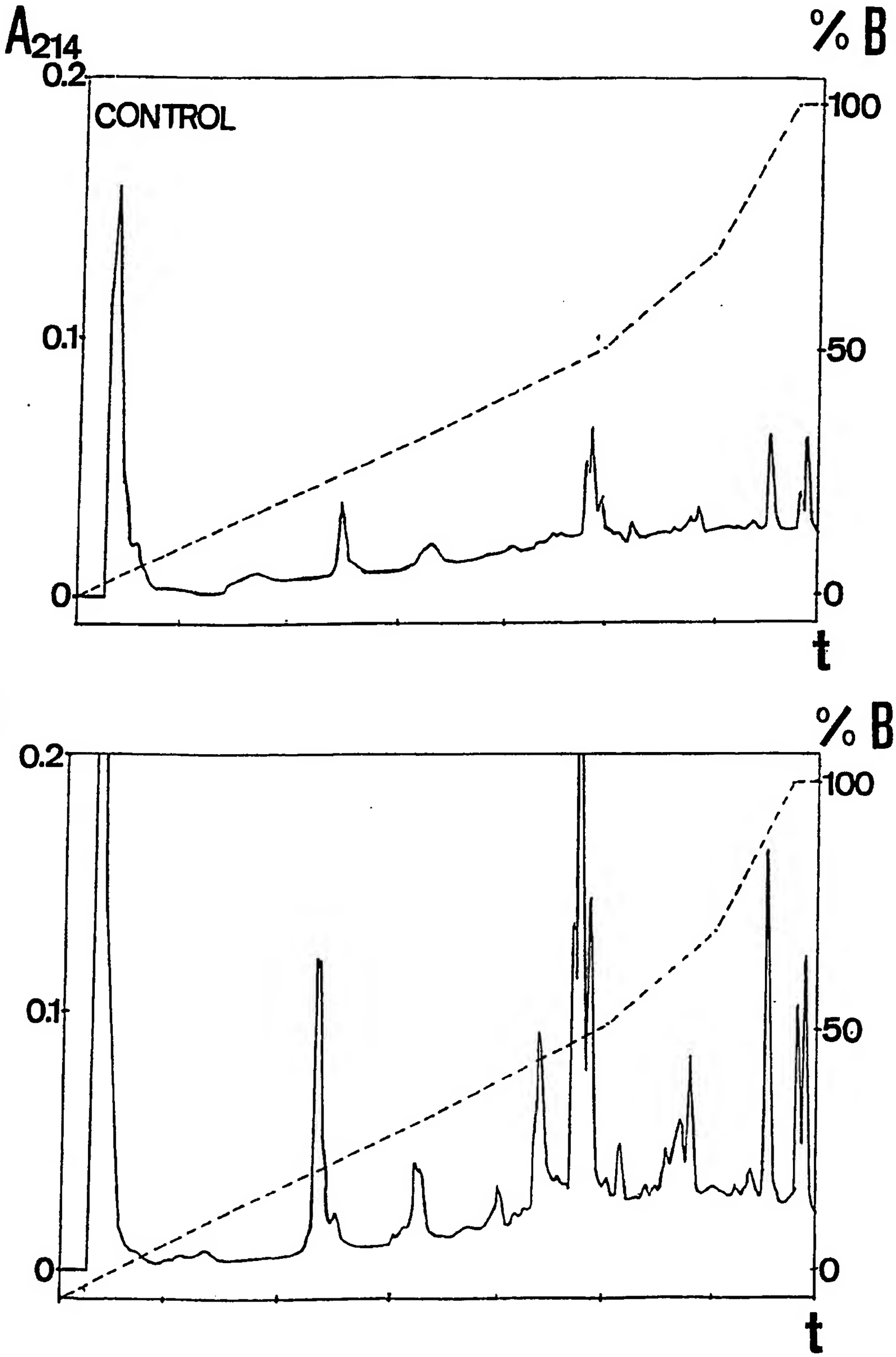
FIG 2



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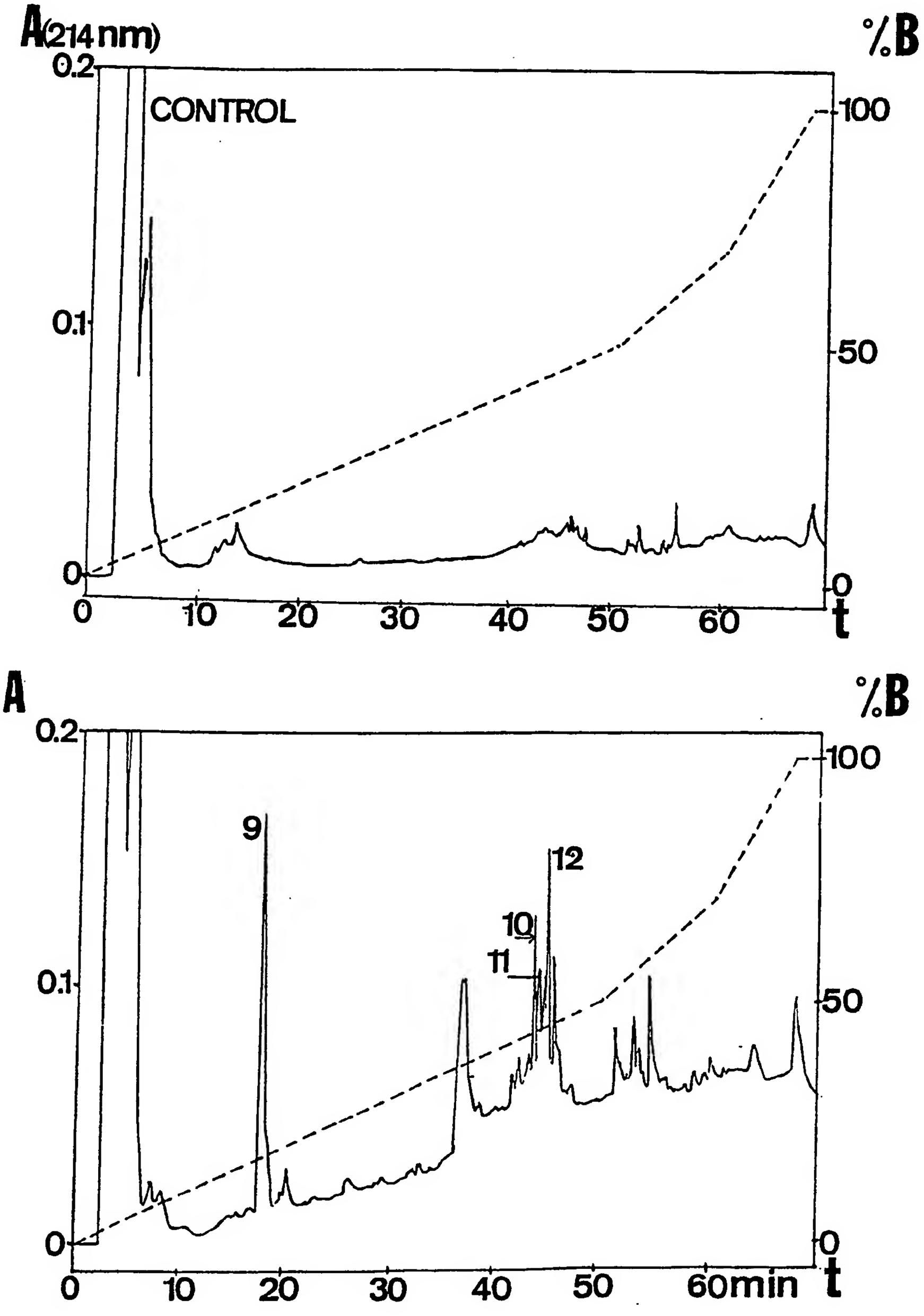
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FIG 4

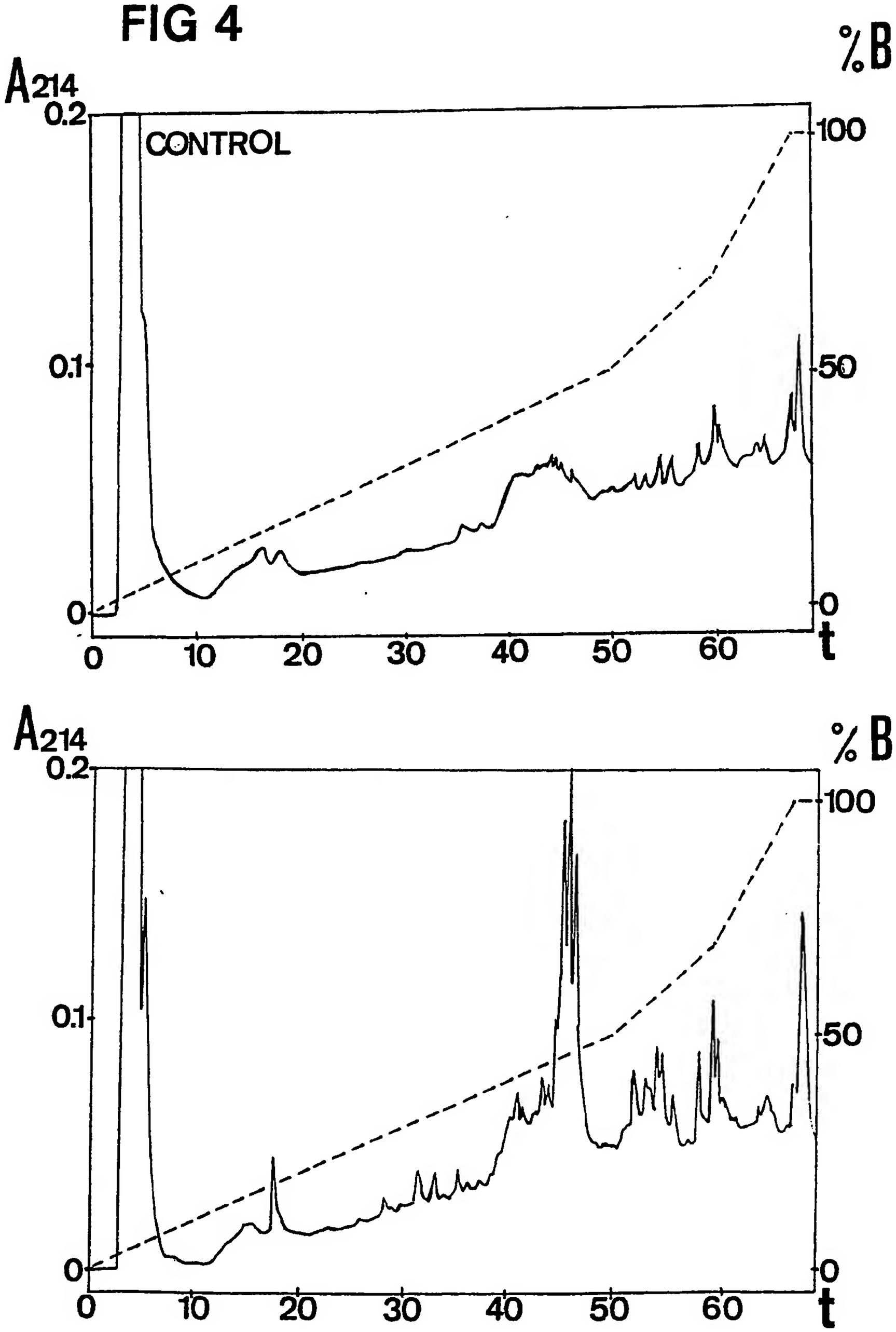
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FIG 5

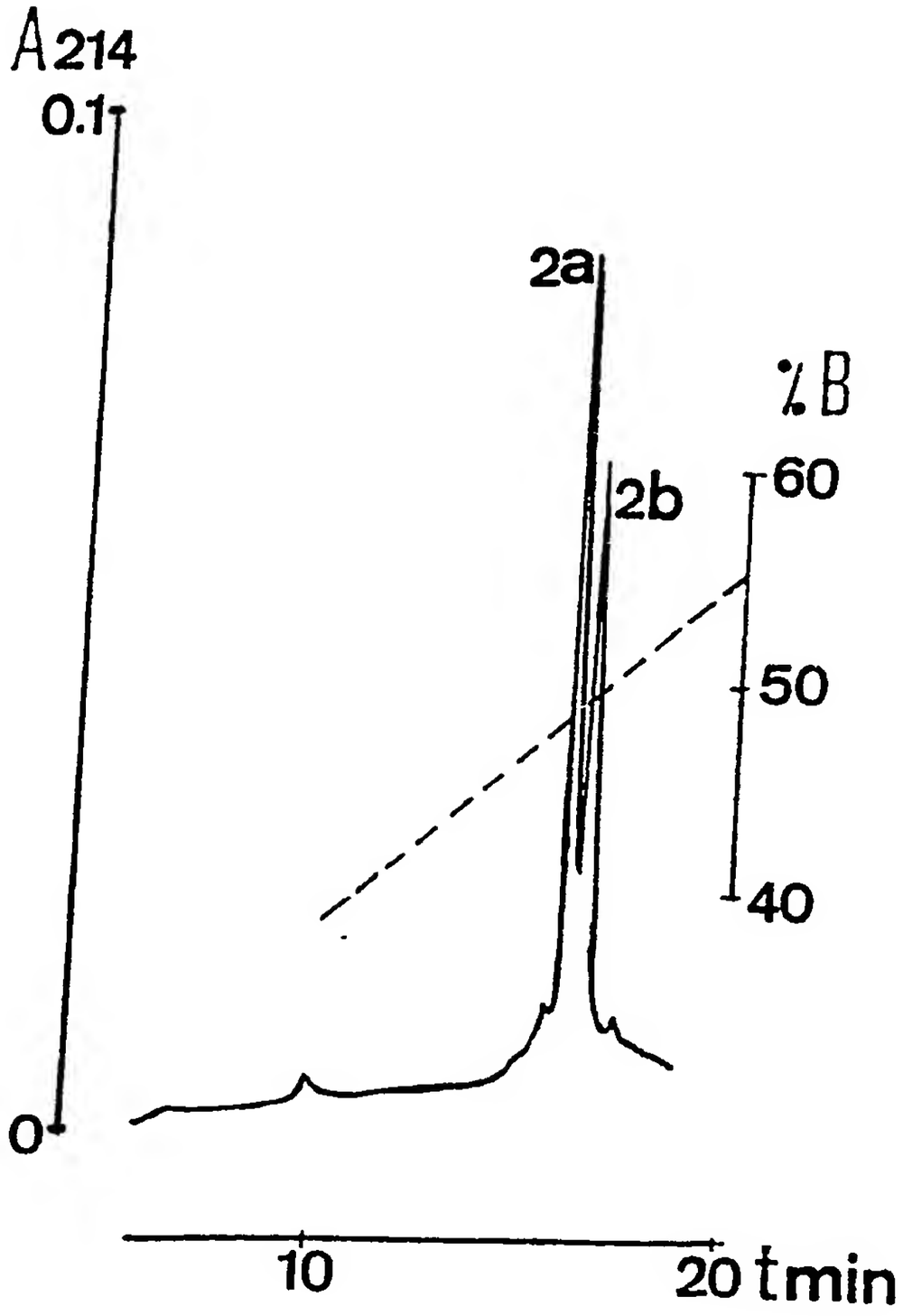
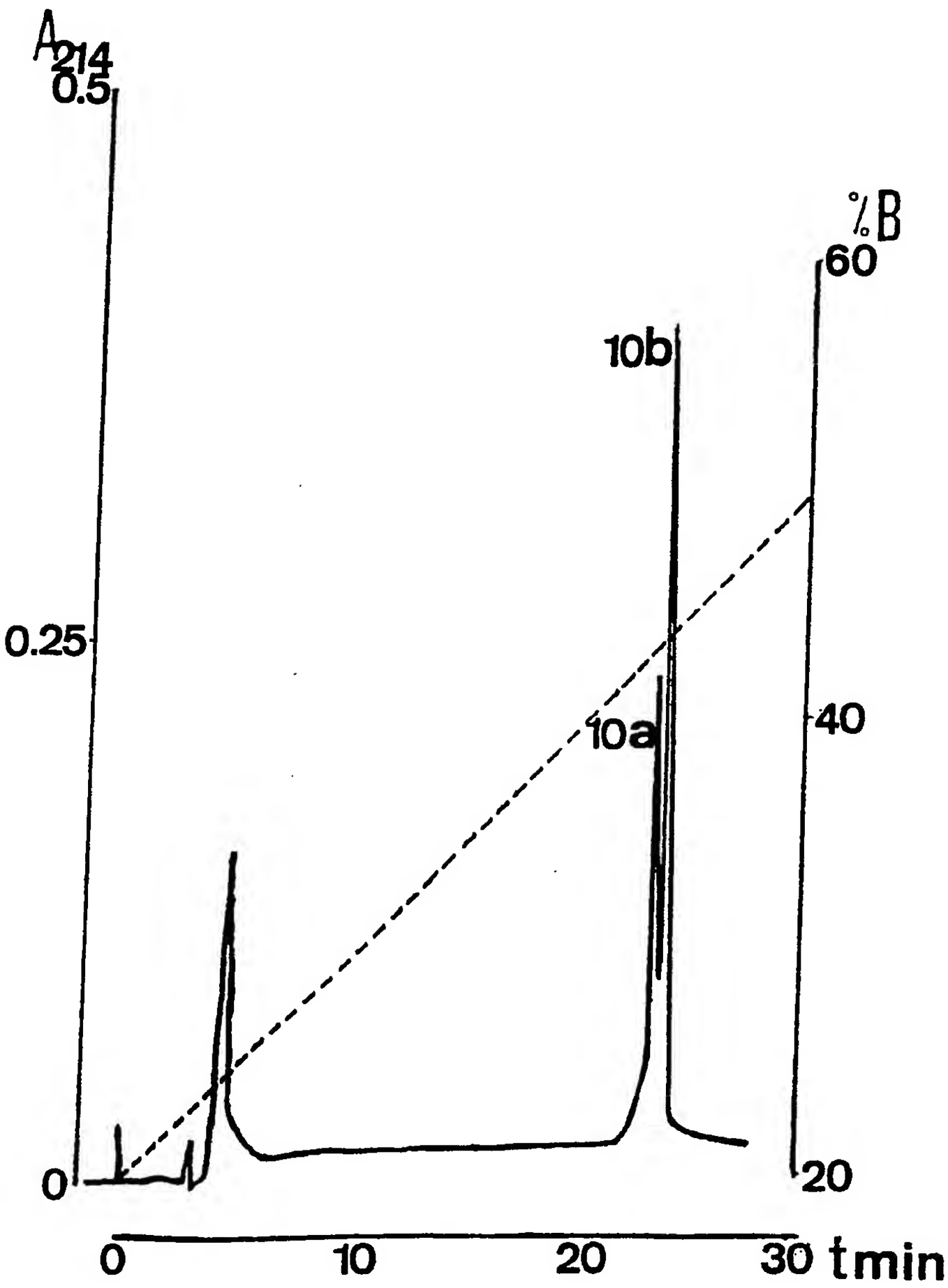


FIG 6



INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/00821

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 35/64, C 12 N 15/82, A 23 L 3/3463, C 07 K 15/08

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K, C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0299828 (PLANT GENETIC SYSTEMS N.V.) 18 January 1989 see the whole document (cited in the application) --	1-8
Y	Chemical Abstracts, vol. 93, no. 13, 29 September 1980, (Columbus, Ohio, US), D.D. Ourth et al.: "Phagocytic and humoral immunity of the adult cotton boll weevil, Anthonomus grandis (Coleoptera: Curculionidae), to Serratia marcescens", see page 499, abstract 130414u, & J. Invertebr. Pathol. 1980, 36(1), 104-12 ----------	1-8

* Special categories of cited documents: [10]

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"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th August 1990	- 1. 10. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. Peis M. PEIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9000821 SA 36974

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/09/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0299828	18-01-89	AU-A- 2121188	30-01-89
		WO-A- 8900048	12-01-89
		JP-T- 2500084	18-01-90